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(21) International Application Number: PCT/US97/09604 (22) International Filing Date: 30 May 1997 (30.05.97) (30) Priority Data: 08/655,551 30 May 1996 (30.05.96) US (71) Applicant (for all designated States except US): CLEVELAND CLINIC FOUNDATION [US/US]; 9500 Euclid Avenue, Cleveland, OH 44195 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): XU, Yan [CA/US]; 6509 Marsol Road #425, Mayfield Heights, OH 44124 (US). CASEY, Graham [GB/US]; 55 Skyline Drive, Moreland Hills, OH 44022 (US). (74) Agents: LONGMUIR, Jeanne, E. et al.; Calfee, Halter & Griswold LLP, 1400 McDonald Investment Center, 800 Superior Avenue, Cleveland, OH 44114-2688 (US).		(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
(54) Title: METHOD OF DETECTING GYNECOLOGICAL CARCINOMAS		
(57) Abstract <p>The present invention provides a new method for detecting the presence of gynecological carcinomas in a patient. The method, which comprises detecting the presence of lysophosphatidic acid in a plasma sample of the patient, is useful for detecting ovarian carcinoma, cervical carcinoma, endometrial carcinoma, and peritoneal carcinoma. In a preferred embodiment the method comprises: providing a blood specimen from the patient, obtaining a plasma sample from the blood specimen under conditions which minimize the release of lysophosphatidic acid from the platelets in the blood specimen into the plasma, extracting lipids from the plasma, and detecting the presence of lysophosphatidic acid in the lipid extract. A new method for extracting lysophosphatidic acid from a biological fluid is also provided. The method which comprises acidifying the biological fluid and extracting the lysophosphatidic acid from the acidified sample with an organic solvent is useful for extracting greater than 80 % of the lysophosphatidic acid in the biological fluid.</p>		

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BACKGROUND OF THE INVENTION

Typically, benign ovarian tumors are distinguished from malignant ovarian tumors by surgical procedures such as biopsy of the mass or aspiration of the mass and cytological examination of the cells that are surgically removed from the patient. However, these techniques are highly invasive, expensive, and in the case of aspiration can lead to release of cancerous cells into the peritoneum.

5 The antigenic determinant CA 125, which is a high
molecular weight mucin-like glycoprotein, is the current
serum biomarker of choice for screening for ovarian
carcinomas. However, CA 125 testing suffers from two main
limitations. First of all, it is not very sensitive. For
example, elevated serum CA 125 levels, i.e. levels above the
cut-off point of 35 U/ml, are present in fewer than 50% of
the patients with Stage I ovarian carcinoma. Taylor, K.J.W.
and Schwartz, P.E., "Screening for Early Ovarian Cancer,"
10 Radiology, 192:1-10, 1994. In addition, CA 125 testing is
not very specific. For example, approximately 25% of
patients with benign gynecological diseases also have
elevated serum levels of CA 125. Moreover, liver disease
such as cirrhosis, even without ascites, elevates serum CA
15 125 levels above 35 U/ml. Taylor, K.J.W. and Schwartz, P.E.,
"Screening for Early Ovarian Cancer," Radiology, 192:1-10,
1994.

 Accordingly, it would be desirable to have a new,
simple, noninvasive or marginally invasive method for
20 detecting gynecological carcinomas, particularly ovarian
carcinomas, which is sufficiently sensitive to identify those
subjects with early stage ovarian carcinoma, and sufficiently
specific to distinguish between benign and malignant
gynecological carcinomas.

25 SUMMARY OF THE INVENTION

 The present invention provides a new, simple,
marginally invasive method for detecting the presence of
gynecological carcinomas in a patient. The method comprises
30 detecting the presence of lysophosphatidic acid in a plasma
sample of the patient.

 The preferred method comprises: providing a blood

specimen from the patient, obtaining a blood plasma sample which is substantially free of platelets from the blood specimen, preparing a lipid extract from said plasma sample, and detecting the presence of lysophosphatidic acid in said lipid extract. Because the method is sufficiently sensitive to detect ovarian carcinoma in subjects with early stage ovarian carcinoma, sufficiently specific to distinguish benign gynecological carcinomas from malignant gynecological carcinomas, and marginally invasive, the method is especially useful for screening patients for ovarian carcinomas.

The method also detects the presence of endometrial carcinoma, peritoneal carcinoma, and cervical carcinoma in a subject. Accordingly, the method is useful for screening for more than one gynecological carcinoma.

A new method for extracting lysophosphatidic acid from a biological fluid is also provided. The method which comprises acidifying the biological fluid and extracting the lysophosphatidic acid from the acidified sample with an organic solvent is useful for extracting greater than 80% of the lysophosphatidic acid in the biological fluid.

DETAILED DESCRIPTION OF THE INVENTION

5 The present invention provides a new, simple method for detecting the presence of a gynecological carcinoma particularly ovarian carcinomas in a patient. The method comprises detecting the presence of lysophosphatidic acid in a blood plasma sample from the patient. Preferably, the blood plasma sample is substantially free of platelets. As described herein, a plasma sample is substantially free of platelets when it contains less than 95% of the platelets present in the original blood specimen obtained from the patient. In a preferred embodiment, the method comprises providing a whole blood specimen from the patient, obtaining a plasma sample which is substantially free of platelets from the whole blood specimen, extracting lipids from the blood plasma sample, and detecting the presence of lysophosphatidic acid in the lipid extract.

10 Preferably, the blood plasma sample is obtained under conditions which minimize the release of lysophosphatidic acid from platelets which are present in the whole blood specimen. Such conditions include, for example, collecting the whole blood specimen from the subject in tubes that contain an anti-coagulant. Suitable anti-coagulants include, for example, heparin and chelating agents. It is preferred that the whole blood specimen be collected in the presence of a chelating agent, such as for example ethylenediaminetetraacetic acid (EDTA) or sodium citrate, more preferably EDTA, since chelating agents both reduce phospholipase activity in the sample and prevent clotting of the whole blood specimen.

25 30 The blood plasma typically is obtained by centrifuging the whole blood specimen to pellet the blood cells in the whole blood specimen and collecting the

supernatant, which represents the major portion of the blood plasma in the whole blood specimen.

Preferably, the blood plasma is obtained from the blood specimen by a procedure which comprises a first centrifugation step to provide a pellet of blood cells and platelets and a first supernatant and a second centrifugation step wherein the first supernatant is centrifuged to provide a second pellet of platelets and a plasma supernatant. Preferably the speed of the first centrifugation step is between 400 to 1000 x g, more preferably from about 500 to 650 x g, most preferably from about 550 to about 600 x g. The supernatant is then removed from the tube and centrifuged a second time at a speed of between 6500 to 10000 x g, more preferably from about 7000 to 8500 x g. The two-step centrifugation procedure minimizes the release of lysophosphatidic acid from platelets and thus permits a more accurate determination of lysophosphatidic acid in the sample.

Substantially all of the lipids, particularly the phospholipids, in the plasma sample are then extracted, preferably by a lipid extraction procedure which recovers at least 80% of the lysophosphatidic acid from the plasma sample. More preferably, the lipid extraction procedure recovers at least 85% of the LPA in the plasma sample. An example of a preferred lipid extraction procedure comprises the steps of: acidifying the blood plasma sample; mixing the acidified blood plasma sample with an organic solvent to provide an aqueous phase and an organic phase, wherein the phospholipids preferentially distribute to the organic phase; and recovering the organic phase to provide a lipid extract which contains greater than 80% of the lysophosphatidic acid in the plasma sample. Preferably, hydrochloric acid is used

to acidify the blood plasma sample. Preferably the final concentration of acid in the acidified plasma sample is from about 0.2 N to about 2.0 N, more preferably from about 1.3 N to about 2.0 N.

5 Suitable organic solvents for extracting lysophosphatidic acid from the acidified plasma sample include for example butanol, isopropanol, and mixed organic solvents which comprise a polar organic solvent, such as for example methanol, and a non-polar organic solvent, such as
10 for example chloroform. Preferably the organic solvent is a mixture of methanol and chloroform at a 2:1 ratio.

 The lysophosphatidic acid, also referred to herein as "LPA," is then separated from the other phospholipids in the organic phase using conventional techniques, such as for
15 example, separation by thin-layer chromatography. Preliminary studies in which standards including lysophosphatidylcholine, lysophosphatidylinositol, lysophosphatidylserine, lysophosphatidylethanolamine, and the lysophosphatidic acids, palmitoyl-LPA, oleoyl-LPA and
20 stearoyl-LPA were separated by one-dimensional TLC indicated that the R_f value of LPA was 0.14 for palmitoyl and stearoyl LPA and 0.13 for oleoyl-LPA. The preliminary studies also indicated that each of the LPA species migrated at a rate slower than that of lysophosphatidylcholine and at a rate
25 different from lysophosphatidylinositol, lysophosphatidylserine, and lysophosphatidylethanolamine. Preliminary studies in which the phospholipid standards were subjected to two-dimensional TLC also indicated that LPA prepared in this manner, i.e. by one-dimensional TLC, does
30 not contain any other phospholipids.

 The amount of lysophosphatidic acid in the LPA fraction is then quantified using conventional techniques.

The quantification technique used depends upon the amount of blood specimen provided by the subject. For example, if the size of the blood specimen is 2 ml or less, it is preferred that a quantification technique which is capable of detecting picomole amounts of LPA be used. Suitable techniques for detecting picomole amounts of LPA include, for example, hydrolyzing the isolated LPA fraction and then quantifying the amount of each fatty acid in the hydrolysate by gas chromatography. If the size of the blood specimen is 20 ml or greater, a technique which is capable of detecting nanomole amounts of LPA, such as for example total phosphorous determination in the LPA fraction, is suitable.

The methods disclosed herein are useful for detecting gynecological carcinomas such as cervical carcinoma, endometrial carcinoma, peritoneal carcinoma, and ovarian carcinoma, that is, epithelial ovarian cancer. Epithelial ovarian cancer includes serous tumors, mucinous tumors, endometrioid tumors, clear cell tumors, undifferentiated carcinoma, mixed epithelial tumors, and unclassified epithelial tumors.

The methods disclosed herein are especially useful for assessing the malignancy of an ovarian mass in a patient and for detecting ovarian carcinomas at the early surgical stage of development in a patient, that is Surgical Stage I and Surgical Stage II, as well as at the later stages of development, that is Surgical Stage III and Surgical Stage IV. Surgical stage represents the severity of disease with Stage I being least severe and Stage IV being the most severe. According to criteria established by the International Federation of Gynecology and Obstetrics, the ovarian carcinoma is limited to the ovaries and may or may not include ascites in Stage I ovarian carcinoma. In Stage

II, there is pelvic extension of the carcinoma and possibly extension to the uterus or fallopian tubes. Stage III ovarian carcinoma is characterized by abdominal metastases. In Stage IV, there is distant metastases of the carcinoma outside of the peritoneal cavity.

The methods disclosed herein are simple, marginally invasive, and require only a blood specimen from the subject. Thus, such methods are also useful for screening patients who have not been previously diagnosed as carrying carcinoma, particularly patients who are at risk for gynecological carcinomas, especially ovarian carcinoma. Such patients include women at elevated risk by virtue of a family history of the disease, premenopausal women with anovulatory cycles, and postmenopausal women.

The present invention is further described by the examples which follow. Such examples, however, are not to be construed as limiting in any way either the spirit or the scope of the present invention.

EXAMPLE

Plasma samples were obtained from blood specimens provided by eighty-four female subjects. A whole blood specimen of approximately 2 ml was collected from each of the subjects in a 5 ml vacutainer tube containing 7.2 mg of EDTA. Obtaining a Plasma Sample from the whole blood specimen

The whole blood specimen was centrifuged at 580 x g for 5 minutes to provide a pellet of the blood cells and platelets and a supernatant. The supernatant was transferred to a siliconized microcentrifuge tube and centrifuged for 5 minutes at 8000 x g to provide a second pellet and a plasma supernatant. The plasma supernatant was either processed immediately or stored at -70°C.

Extracting LPA from a Plasma Sample.

Extraction of lipids, particularly LPA from the plasma sample was performed at 0-4° C. Each 1 mL sample of plasma was acidified by adding 0.2 mL of 12 N HCl to provide a plasma sample with a final concentration of HCl of approximately 2.0 N. After mixing, 4 mL of mixed organic solvent comprising a 2:1 ratio of methanol/chloroform was added to the acidified plasma and the mixture was vortexed for 1 minute and incubated on ice for 10 minutes. 1 mL of chloroform and 1.25 mL of H₂O were added to the mixture. After mixing thoroughly, the mixture was centrifuged at 1000 x g for 10 minutes at 4°C. The lower organic layer was transferred to a new glass tube and dried at 40°C under nitrogen to provide a phospholipid extract.

The total recovery rate and reproducibility of this extraction procedure was examined by adding 10 nmol of synthetic oleoyl-LPA to three parallel plasma samples lacking endogenous oleoyl-LPA. The samples were extracted by the present procedure and the amount of oleoyl-LPA in each sample quantified. This analysis indicated that the average recovery of LPA by the present extraction procedure was 92.7% with a standard error of $\pm 5.6\%$.

Isolating the LPA in the Lipid Extract

Each lipid extract was dissolved in 50 μ l of a 2:1 methanol/chloroform mixture and loaded onto a single lane of a precoated silica-gel 60 TLC plate (20 x 20 cm, layer thickness 250 μ m) obtained from EM Science, Darmstadt, Germany. A 50 μ l aliquot of a 2:1 methanol/chloroform mixture containing 10 to 50 μ g of oleoyl-LPA obtained from Avanti Polar-Lipids, Inc. was loaded onto one outside lane of the plate and a 50 μ l aliquot of chloroform containing 10 to 50 μ g of lysophosphatidylcholine obtained from Sigma Chemical Company was loaded onto the opposite outside lane of the plate. The oleoyl-LPA and lysophosphatidylcholine were used as migration references for locating the LPA fractions on the plate. The lipids were developed at room temperature for approximately 3 hours with a mixture of chloroform-methanol-ammonium hydroxide (65:35:5.5). Then the outside lanes containing the oleoyl LPA and lysophosphatidylcholine standards were sprayed with 0.1% 8-anilino-1-naphthalene-sulfonic acid and visualized under ultraviolet light.

Each of the isolated LPA fractions in the lanes loaded with lipid extracts from the plasma samples were scraped from the plates into separate 15 mL disposable glass centrifuge tubes from Kimble, Vineland, New Jersey. The LPA fractions included all of the lipids that migrated to a distance on the plate which corresponded to the R_f of the oleoyl-LPA standard and extended to but was less than the R_f of the lysophosphatidylcholine standard.

Measuring the Amount of LPA Isolated from Each Sample

2 mL of 1M ethanolic KOH were added to each tube containing the isolated LPA fractions. The tubes were then incubated at 60°C for 1 hour to hydrolyze the LPA present in the tube. After cooling, 5 µl of internal standard solution contains methyl behenate, 1.5mg/mL in chloroform, 1 mL of 6N HCl, 5 mL of H₂O and 5 mL of ethyl ether were added to the hydrolysate. The mixture was vortexed for 30 seconds and centrifuged at 1000 x g for 10 minutes. Then, the upper layer was transferred into a new glass tube and dried at 40°C under nitrogen. The residue, which contained fatty acids released from the LPA, was dissolved in 0.6 mL of petroleum ether and the dissolved fatty acids were transmethyalted by adding 1 mL of BCl₃-methanol reagent to each tube and incubating at 100°C for 10 minutes. BCl₃ methanol was obtained from Supelco Inc., Bellefonte, PA. The fatty acid methyl esters were extracted with 1 ml of petroleum ether. The mixture was vortexed for 10 seconds and centrifuged at 1000 x g for 5 minutes. The top layer was transferred to a 3.7 mL screw-topped Supelco sample vial and dried at 40°C under nitrogen. The residue was then dissolved in 25 µl of chloroform and 5-15 µl of the chloroform solution was used for analysis by gas chromatography.

GC analysis of the Fatty Acids Released from LPA by Hydrolysis

A Hewlett-Packard Model 5710A gas chromatograph, equipped with a fused silica column (25 m x 0.2 mm) coated with 3% SP-2310, 2% SP-2300 on 100/120 Chromosorb WAW from Supelco Inc., was used to measure the amount of each fatty acid in each of the samples. The gas chromatograph conditions were as follows: the oven starting temperature was 185° C; after 2 minutes, the temperature was increased at 2°C/minutes to 230°C, and held for 4 minutes; the injector temperature was 200°C; the detector temperature was 300°C; nitrogen was the carrier gas at a flow rate of 30ml/minutes at 50 PSI; air flow rate was 240ml/minutes at 24 PSI; hydrogen flow was 30ml/min at 15 Psi. A flame ionization detector was used to detect the fatty acid methyl esters. Two standard curves were obtained using two fatty acid methyl esters standard mixtures obtained from Nu Check Prep. Inc., Elysian, MN. The two standard mixtures included different combinations of the esters of methyl palmitate, methyl stearate, methyl oleate, methyl linoleate, methyl arachidonate and methyl behenate. The retention times were 3.8 min for methyl palmitate, 6.7 min for methyl stearate, 7.3 min for methyl oleate, 8.3 min for methyl linoleate, 14.0 min for methyl arachidonate and 15.3 min for methyl behenate (internal standard).

The concentrations of each fatty acid in each sample were calculated and added together to obtain the concentration of total LPA in each plasma sample. The concentrations in μM of each LPA species and of total LPA in each of the samples are presented in Table I.

Each of the female subjects also underwent one or more routine diagnostic procedures to determine whether she was healthy or had an active disease. The diagnostic procedures included, where appropriate, clinical examination, clinical chemistries, and surgical evaluation of any masses detected. On the basis of these routine diagnostic procedures, the patients were diagnosed as being healthy or as having an active form of one of the diseases listed in Table I.

Table I
LPA Levels (μ M) in Plasma of Subjects of the Example

Diagnosis	LPA(P)	LPA(S)	LPA(O)	LPA(L)	LPA(A)	LPA(D)	TOTAL LPA
1. Ovarian Carcinoma Stage I							
#1	9.97	12.84	4.39	2.83	2.29	B.D.	32.32
#2	10.78	10.28	1.84	B.D.	B.D.	B.D.	22.9
#3	1.39	0.40	0.87	2.01	B.D.	B.D.	4.67
2. Ovarian Carcinoma Stage II							
#1	0.78	2.17	0.68	0.98	0.41	B.D.	5.02
3. Ovarian Carcinoma Stage III							
#1	8.87	9.15	3.98	4.17	3.32	B.D.	29.49
#2	7.90	6.97	3.46	8.97	6.45	B.D.	33.75
#3	9.59	6.96	1.59	1.64	2.56	B.D.	22.34
#4	11.09	7.62	5.72	11.78	6.85	B.D.	43.06
#5	5.64	4.14	B.D.	B.D.	B.D.	B.D.	9.78
#6	3.10	4.62	1.58	3.18	1.59	B.D.	14.07
#7	2.13	1.80	0.39	0.91	B.D.	B.D.	5.23
#8	0.81	1.56	0.77	B.D.	B.D.	B.D.	3.14
#9	3.30	6.22	2.28	5.40	5.17	B.D.	22.37
#10	2.37	4.33	1.56	0.77	0.92	B.D.	9.95
#11	5.34	8.32	2.68	1.85	2.39	B.D.	20.58
#12	8.97	10.23	11.39	13.50	8.96	3.12	56.17
#13	1.08	0.89	0.31	0.25	B.D.	B.D.	2.53
#14	B.D.	1.82	0.43	2.42	2.42	B.D.	5.84
4. Ovarian Carcinoma Stage IV							
#1	3.61	4.91	2.09	1.16	B.D.	B.D.	11.77
5. Peritoneal Carcinoma							
#1	14.22	9.93	3.08	3.79	1.94	B.D.	32.96

Table I, Continued

Diagnosis	LPA(P)	LPA(S)	LPA(O)	LPA(L)	LPA(A)	LPA(D)	TOTAL LPA
#2	3.19	6.29	0.68	6.23	B.D.	B.D.	16.41
#3	2.65	2.29	0.13	B.D.	B.D.	B.D.	5.07
#4	12.81	11.45	12.50	14.92	9.19	2.34	63.21
#5	0.70	1.17	B.D.	B.D.	B.D.	B.D.	1.87
#6	B.D.	B.D.	B.D.	B.D.	B.D.	B.D.	B.D.
#7	3.89	4.01	3.94	4.51	2.84	B.D.	19.19
#8	3.11	3.15	1.72	2.40	1.70	B.D.	12.08
#9	1.16	B.D.	B.D.	B.D.	0.28	B.D.	1.44
6. Endometrial Carcinoma							
#1	8.46	9.45	3.85	4.83	2.23	B.D.	28.82
#2	11.01	11.31	2.33	1.03	B.D.	B.D.	25.68
#3	6.84	15.84	12.62	18.88	9.05	B.D.	63.23
#4	7.68	7.27	3.53	5.67	7.24	B.D.	31.39
#5	1.71	1.65	0.59	B.D.	B.D.	B.D.	3.95
#6	6.19	7.65	2.59	5.27	2.89	B.D.	24.59
#7	21.15	5.16	16.41	20.98	B.D.	B.D.	54.56
7. Cervical Carcinoma							
#1	5.94	5.60	2.99	1.19	2.10	B.D.	17.82
#2	12.45	11.18	6.72	10.44	6.53	B.D.	47.32
#3	0.86	1.14	B.D.	B.D.	B.D.	B.D.	2.00
#4	15.29	12.98	8.27	5.68	8.97	B.D.	B.D.
8. Healthy							
#1	B.D.	B.D.	B.D.	B.D.	B.D.	B.D.	B.D.
#2	B.D.	B.D.	0.42	B.D.	B.D.	B.D.	0.42
#3	B.D.	B.D.	B.D.	B.D.	B.D.	B.D.	B.D.
#4	B.D.	B.D.	B.D.	B.D.	B.D.	B.D.	B.D.
#5	B.D.	B.D.	B.D.	B.D.	B.D.	B.D.	B.D.
#6	B.D.	1.94	0.21	B.D.	B.D.	B.D.	2.15
#7	B.D.	B.D.	B.D.	B.D.	B.D.	B.D.	B.D.
#8	B.D.	B.D.	B.D.	B.D.	B.D.	B.D.	B.D.
#9	B.D.	B.D.	B.D.	B.D.	B.D.	B.D.	B.D.

Table I, Continued

Diagnosis	LPA(P)	LPA(S)	LPA(O)	LPA(L)	LPA(A)	LPA(D)	TOTAL LPA
#10	B.D.	B.D.	B.D.	B.D.	B.D.	B.D.	B.D.
#11	B.D.	B.D.	B.D.	B.D.	B.D.	B.D.	B.D.
#12	B.D.	B.D.	B.D.	B.D.	B.D.	B.D.	B.D.
#13	B.D.	B.D.	0.42	B.D.	B.D.	B.D.	0.42
#14	B.D.	B.D.	B.D.	B.D.	B.D.	B.D.	B.D.
#15	B.D.	B.D.	B.D.	B.D.	B.D.	B.D.	B.D.
#16	B.D.	B.D.	B.D.	B.D.	B.D.	B.D.	B.D.
#17	0.48	0.69	B.D.	B.D.	B.D.	B.D.	1.17
#18	B.D.	B.D.	B.D.	B.D.	B.D.	B.D.	B.D.
#19	B.D.	B.D.	B.D.	B.D.	B.D.	B.D.	B.D.
#20	B.D.	B.D.	B.D.	B.D.	B.D.	B.D.	B.D.
#21	B.D.	B.D.	B.D.	B.D.	B.D.	B.D.	B.D.
#22	4.93	B.D.	B.D.	B.D.	B.D.	B.D.	4.93
#23	B.D.	B.D.	B.D.	B.D.	B.D.	B.D.	B.D.
9. Benign GYN Diseases							
#1	B.D.	B.D.	B.D.	B.D.	B.D.	B.D.	B.D.
#2	B.D.	B.D.	B.D.	B.D.	B.D.	B.D.	B.D.
#3	B.D.	B.D.	B.D.	B.D.	B.D.	B.D.	B.D.
10. Sarcoma							
#1	B.D.	B.D.	B.D.	B.D.	B.D.	B.D.	B.D.
#2	B.D.	B.D.	B.D.	B.D.	B.D.	B.D.	B.D.
#3	B.D.	B.D.	B.D.	B.D.	B.D.	B.D.	B.D.
#4	B.D.	2.08	0.99	1.43	3.48	B.D.	7.97
11. Breast Cancer							
#1	B.D.	B.D.	B.D.	B.D.	B.D.	B.D.	B.D.
#2	B.D.	B.D.	0.42	B.D.	B.D.	B.D.	0.42
#3	B.D.	B.D.	B.D.	B.D.	B.D.	B.D.	B.D.
#4	B.D.	B.D.	B.D.	B.D.	B.D.	B.D.	B.D.
#5	B.D.	B.D.	B.D.	B.D.	B.D.	B.D.	B.D.
#6	B.D.	B.D.	B.D.	B.D.	B.D.	B.D.	B.D.
#7	B.D.	B.D.	B.D.	B.D.	B.D.	B.D.	B.D.

Table I, Continued

Diagnosis	LPA(P)	LPA(S)	LPA(O)	LPA(L)	LPA(A)	LPA(D)	TOTAL LPA
#8	B.D.	B.D.	B.D.	B.D.	B.D.	B.D.	B.D.
#9	B.D.	B.D.	B.D.	B.D.	B.D.	B.D.	B.D.
#10	B.D.	B.D.	B.D.	B.D.	B.D.	B.D.	B.D.
#11	B.D.	B.D.	B.D.	B.D.	B.D.	B.D.	B.D.
12. Leukemia							
#1	B.D.	B.D.	B.D.	B.D.	B.D.	B.D.	B.D.
#2	B.D.	B.D.	B.D.	B.D.	B.D.	B.D.	B.D.
#3	B.D.	B.D.	B.D.	B.D.	B.D.	B.D.	B.D.
#4	B.D.	B.D.	B.D.	B.D.	B.D.	B.D.	B.D.

LPA(P): palmitoyl - LPA
 LPA(S): stearoyl - LPA
 LPA(O): oleoyl - LPA
 LPA(L): linolenyl - LPA
 LPA(A): arachidyl - LPA
 LPA(D): docosahexanyl - LPA

B.D.: below detection

N.A.: Not available

5 The average concentrations \pm SE of each LPA species in
the plasma of patients diagnosed as having a gynecological
carcinoma and the average concentration \pm SE of each LPA
species in the plasma of patients diagnosed as not having a
gynecological carcinoma are presented in Table II. The
average concentrations \pm SE of total LPA in the plasma of
patients with the gynecological carcinomas and the average
10 concentrations \pm SE of total LPA in the plasma of patients
without gynecological carcinomas are presented in Table III.
The statistical power calculations were performed using the
Wilcoxon Rank Sum statistical test as described by W.J.
Conover in Practical Nonparametric. 0.1 μ m was used in
15 calculations where LPA levels were below detection.

Table II

**Average Concentration of Individual LPA Species
in the Plasma of Subjects from the Example**

Diagnosis	N	LPA	Mean	SE	SD	Median	Min	Max
Healthy	23	LPA(P)	0.33	0.21	1.01	0.10	0.10	4.93
		LPA(S)	0.21	0.08	0.40	0.10	0.10	1.94
		LPA(O)	0.10	0.00	0.02	0.10	0.10	0.21
		LPA(L)	0.10	0.00	0.00	0.10	0.10	0.10
		LPA(A)	0.10	0.00	0.00	0.10	0.10	0.10
		LPA(D)	0.10	0.00	0.00	0.10	0.10	0.10
Ovarian Carcinoma	19	LPA(P)	5.10	0.88	3.85	3.61	0.10	11.09
		LPA(S)	5.54	0.83	3.61	4.91	0.40	12.84
		LPA(O)	2.43	0.61	2.66	1.59	0.10	11.39
		LPA(L)	3.27	0.91	3.96	1.85	0.10	13.50
		LPA(A)	2.32	0.62	2.70	1.59	0.10	8.96
		LPA(D)	0.26	0.16	0.69	0.10	0.10	3.12
Peritoneal Carcinoma	9	LPA(P)	4.65	1.73	5.19	3.11	0.10	14.22
		LPA(S)	4.28	1.38	4.14	3.15	0.10	11.45
		LPA(O)	2.48	1.34	4.02	0.68	0.10	12.50
		LPA(L)	3.59	1.61	4.83	2.40	0.10	14.92
		LPA(A)	1.82	0.98	2.95	0.28	0.10	9.19
		LPA(D)	0.35	0.25	0.75	0.10	0.10	2.34
Endometrial Carcinoma	7	LPA(P)	9.01	2.29	6.05	7.68	1.71	21.15
		LPA(S)	8.33	1.71	4.52	7.65	1.65	15.84
		LPA(O)	5.99	2.27	6.02	3.53	0.59	16.41
		LPA(L)	8.11	3.16	8.37	5.27	0.10	20.98

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Diagnosis	N	LPA	Mean	SE	SD	Median	Min	Max
		LPA(A)	3.10	1.38	3.66	2.23	0.10	9.05
		LPA(D)	0.10	0.00	0.00	0.10	0.10	0.10
Cervical Carcinoma	4	LPA(P)	8.64	3.25	6.50	9.20	0.86	15.29
		LPA(S)	7.73	2.70	5.40	8.39	1.14	12.98
		LPA(O)	4.52	1.84	3.69	4.86	0.10	8.27
		LPA(L)	4.35	2.36	4.72	3.44	0.10	10.44
		LPA(A)	4.43	2.02	4.05	4.32	0.10	8.97
		LPA(D)	0.10	0.00	0.00	0.10	0.10	0.10
Benign Gynecological Disease	3	LPA(P)	0.10	0.00	0.00	0.10	0.10	0.10
		LPA(S)	0.10	0.00	0.00	0.10	0.10	0.10
		LPA(O)	0.10	0.00	0.00	0.10	0.10	0.10
		LPA(L)	0.10	0.00	0.00	0.10	0.10	0.10
		LPA(A)	0.10	0.00	0.00	0.10	0.10	0.10
		LPA(D)	0.10	0.00	0.00	0.10	0.10	0.10
Sarcoma	4	LPA(P)	0.10	0.00	0.00	0.10	0.10	0.10
		LPA(S)	0.60	0.50	0.99	0.10	0.10	2.08
		LPA(O)	0.32	0.22	0.45	0.10	0.10	0.99
		LPA(L)	0.43	0.33	0.67	0.10	0.10	1.43
		LPA(A)	0.95	0.85	1.69	0.10	0.10	3.48
		LPA(D)	0.10	0.00	0.00	0.10	0.10	0.10
Breast Cancer	11	LPA(P)	0.10	0.00	0.00	0.10	0.10	0.10
		LPA(S)	0.10	0.00	0.00	0.10	0.10	0.10
		LPA(O)	0.18	0.03	0.10	0.10	0.10	0.43
		LPA(L)	0.10	0.00	0.00	0.10	0.10	0.10

Diagnosis	N	LPA	Mean	SE	SD	Median	Min	Max
		LPA(A)	0.10	0.00	0.00	0.10	0.10	0.10
		LPA(D)	0.10	0.00	0.00	0.10	0.10	0.10
Leukemia	4	LPA(P)	0.10	0.00	0.00	0.10	0.10	0.10
		LPA(S)	0.10	0.00	0.00	0.10	0.10	0.10
		LPA(O)	0.10	0.00	0.00	0.10	0.10	0.10
		LPA(L)	0.10	0.00	0.00	0.10	0.10	0.10
		LPA(A)	0.10	0.00	0.00	0.10	0.10	0.10
		LPA(D)	0.10	0.00	0.00	0.10	0.10	0.10

LPA(P): palmitoyl - LPA
 LPA(S): stearoyl - LPA
 LPA(O): oleoyl - LPA
 LPA(L): linolenyl - LPA
 LPA(A): arachidyl - LPA
 LPA(D): docosahexanyl - LPA

B.D.: below detection

N.A.: Not available

Table III
Average Concentration of Total LPA (μ M) in the Plasma of Patients

Diagnosis	N	Mean	SE	SD	Median	Min	Max
Healthy	23	0.45	0.23	1.09	0.10	0.10	4.93
Ovarian Carcinoma	19	18.68	3.43	14.97	14.07	2.53	56.17
Peritoneal Carcinoma	9	16.93	6.79	20.37	12.08	0.10	63.21
Endometrial Carcinoma	7	33.17	7.51	19.86	28.82	3.95	63.23
Cervical Carcinoma	4	29.58	11.83	23.67	32.57	2.00	51.19
Benign Gynecological Disease	3	0.10	0.00	0.00	0.10	0.10	0.10
Sarcoma	4	4.83	1.05	5.04	3.62	0.10	19.92
Breast Cancer	11	0.13	0.03	0.10	0.10	0.10	0.43
Leukemia	4	0.10	0.00	0.00	0.10	0.10	0.10

LPA(P): palmitoyl - LPA
 LPA(S): stearoyl - LPA
 LPA(O): oleoyl - LPA
 LPA(L): linolenyl - LPA
 LPA(A): arachidyl - LPA
 LPA(D): docosahexanyl - LPA

B.D.: below detection

N.A.: Not available

As shown in Table I, detectable levels of total LPA were found in each of the patients with I, Stage II, Stage III or Stage IV ovarian carcinoma. Thus, every one of the patients diagnosed as having early stage ovarian carcinoma or late state ovarian carcinoma had detectable levels of LPA in her plasma. No false negatives were observed in any of the patients with ovarian carcinoma. The concentration of total LPA in subjects with Stage I and Stage II ovarian carcinoma ranged from 4.67 to 32.32 μM . The concentration of total LPA in subjects with Stage III and Stage IV carcinoma ranged from 2.53 to 56.17 μM . Moreover, detectable levels of stearyl-LPA were found in all of the patients with ovarian carcinoma and detectable levels of the LPA species palmitoyl-LPA and oleoyl-LPA were found in 18 out of the 19 patients with ovarian carcinoma.

In contrast, 19 out of the 23 subjects who were characterized as being healthy, did not have detectable levels of LPA in their plasma. The remaining four healthy patients had low concentrations of LPA in their plasma, ranging from 0.42 to 4.93 μM . It is not known whether these values represent false positives or whether these individuals had a gynecological carcinoma that was not detected by the other routine procedures used to diagnose the patients. Moreover, the average concentration of total LPA and of each LPA species in the plasma of patients diagnosed as having ovarian carcinoma was significantly higher than the average concentration of total LPA and of each LPA-species in the plasma of healthy patients. These results establish that the present method is highly sensitive and therefore useful for identifying those patients with the gynecological carcinoma of ovarian carcinoma, including those patients whose ovarian

carcinomas are still in the early stages of development. The results also indicate that a method which detects the presence of palmitoyl LPA or stearoyl-LPA or oleoyl-LPA or combinations thereof in the plasma of patients is also useful for detecting ovarian carcinomas.

LPA was also detected in the plasma of every one of the subjects diagnosed as having cervical carcinoma and endometrial carcinoma, and in eight out of the nine subjects diagnosed as having peritoneal carcinoma. The peritoneal carcinoma had been totally debulked in the one patient who did not have detectable levels of LPA in her plasma. This result suggests that LPA is not present in the plasma of patients whose carcinomas are completely removed by surgery. Thus, the present method is useful for monitoring the recurrence of gynecological carcinomas in patients who have undergone surgical removal of the carcinoma. The concentration of LPA in the plasma of the subjects with cervical carcinoma and endometrial carcinoma ranged from 2.00 to 63.23 μM .

In contrast, detectable levels of LPA were not present in 15 out of the 17 patients diagnosed as having a cancer other than a gynecological carcinoma, i.e., breast cancer, leukemia, and uterine sarcoma. Moreover, detectable levels of LPA were not present in any of the subjects with uterine fibroids, a benign gynecological disease. Since plasma from three out of the four patients with gynecological sarcomas, which are derived from connective tissues, and with benign uterine fibroids did not have detectable levels of total LPA in their plasma, it is believed that malignant epithelial cells of the respective gynecological organ may be the source of the LPA in the plasma of patients with ovarian carcinoma, cervical carcinoma, endometrial carcinoma, and peritoneal carcinoma.

Because of its sensitivity, simplicity, and low cost, the present method is useful for screening patients for gynecological carcinomas. Because the blood specimens for the present method and for CA 125 testing can be drawn from a patient at the same time, CA 125 testing can also be performed when patients are screened for gynecological carcinomas by the present methods. Alternatively, the present method can be used alone to detect gynecological carcinomas.

Because the LPA is not present in the plasma of patients with benign gynecological diseases, the present method is also useful for distinguishing between gynecological diseases which are benign and gynecological diseases which are malignant in patients with gynecological masses. Use of this method to discriminate between malignant and benign ovarian masses should reduce the number of patients required to undergo the more expensive techniques such as transvaginal sonography or the more invasive techniques such as tumor biopsy or tumor aspiration that are now used to diagnose ovarian tumor malignancy.

Although the invention has been described with regard to a number of preferred embodiments, which constitute the best mode presently known to the inventors for carrying out this invention, it should be understood that various changes and modifications as would be obvious to one having the ordinary skill in this art may be made without departing from the scope of the invention which is defined by the claims which are appended hereto.

What is claimed:

1 1. A method of detecting the presence of a gynecological
2 carcinoma in a subject comprising the following steps:
3 (a) providing a blood specimen from the subject;
4 (b) preparing a plasma sample from said blood
5 specimen; and
6 (c) detecting the presence of lysophosphatidic
7 acid in said plasma sample.

1 2. The method of claim 1 wherein the gynecological carcinoma
2 is selected from the group consisting of ovarian carcinoma,
3 peritoneal carcinoma, endometrial carcinoma, cervical
4 carcinoma, and combinations thereof.

1 3. The method of claim 1 wherein said plasma sample is
2 prepared from said blood specimen by a process which
3 comprises the following steps:
4 (a) centrifuging said blood specimen at a first
5 speed of between 400 x g and 1000 x g to obtain a
6 first pellet of blood cells and platelets and a
7 first supernatant; and
8 (b) centrifuging the first supernatant of step (a)
9 at a second speed of between 6500 and 10000 x g
10 to obtain a second pellet and a second
11 supernatant;
12 (c) collecting said second supernatant from step
13 (b) to provide said plasma sample.

1 4. The method of claim 1 wherein the presence of
2 lysophosphatidic acid in said plasma sample is detected by a
3 process which comprises the following steps:

- 4
5 (a) preparing a lipid extract from said plasma
- 6 sample;
7 (b) detecting the presence of lysophosphatidic
8 acid in said lipid extract.

1 5. The method of claim 4 wherein the presence of
2 lysophosphatidic acid in said lipid extract is detected by a
3 process which comprises the following steps:

- 4 (a) separating said lipid extract into
5 phospholipid fractions; and
6 (b) detecting the presence of lysophosphatidic
7 acid
8 in said phospholipid fractions.

1 6. The method of claim 4 wherein the presence of
2 lysophosphatidic acid in said lipid extract is detected by a
3 process which comprises the following steps:

- 4
5 (a) separating said lipid extract into
6 phospholipid fractions; and
7 (b) detecting the presence of palmitoyl-LPA, or
8 stearyl-LPA, or oleoyl-
9 LPA or combinations thereof
10 in said phospholipid fractions.

1 7. The method of claim 1 wherein said gynecological
2 carcinoma is ovarian carcinoma.

1 8. The method of claim 3 wherein said gynecological
2 carcinoma is ovarian carcinoma.

1 9. The method of claim 4 wherein said gynecological
2 carcinoma is ovarian carcinoma.

1 10. The method of claim 5 wherein said gynecological
2 carcinoma is ovarian carcinoma.

1 11. A method for extracting LPA from a biological fluid
2 sample comprising the following steps:

3
4 (a) acidifying said biological fluid sample with
5 a sufficient amount of acid to produce an acidified
6 sample wherein the final concentration of acid in
7 said sample is from about 0.2 to 2.0 N.

8 (b) mixing said acidified sample with an organic
9 solvent to provide an aqueous phase and an organic
10 phase;

11 (c) separating said aqueous phase from said
12 organic phase to provide an organic phase extract
13 containing greater than 80% of the LPA in said
14 biological fluid sample.

1 12. The method of claim 11 wherein said biological fluid
2 sample is a blood plasma sample substantially free of
3 platelets.

1 13. The method of claim 11 wherein the final concentration
2 of acid in said acidified sample is from about 1.3 to about
3 2.0 N.

- 1 14. A method for detecting a gynecological carcinoma in a
2 subject comprising the following steps:
3 (a) providing a plasma sample substantially free
4 of platelets from the subject;
5 (b) preparing a lipid extract from said plasma
6 sample; and
7 (c) detecting the presence of lysophosphatidic
8 acid in said lipid extract.
- 1 15. The method of claim 14 wherein said gynecological
2 carcinoma is ovarian carcinoma.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/09604

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : G01N 33/48, 33/92, 30/02, 30/90, 1/18, 33/00

US CL : 436/63, 64, 71, 161, 162, 177, 103, 104, 105

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 436/63, 64, 71, 161, 162, 177, 103, 104, 105

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, BIOSIS, CAS ONLINE, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X ----- Y	EICHHOLTZ et al, The Bioactive Phospholipid Lysophosphatidic Acid is Released From Activated Platelets, Biochem. J., 1993, Vol. 291, 677-680, especially pages 677-678.	1, 4-6, 14 ----- 3, 11-12
Y	XU et al, Characterization of an Ovarian Cancer Activating Factor in Ascites from Ovarian Cancer Patients. Clinical Cancer Research, October 1995, Vol. 1, pages 1223-1232, especially pages 1224, 1226-1227, 1230.	2, 7-11
Y	US 5,277,917 A (XU et al) 11 January 1994, col. 2, lines 50-65, col. 3, lines 25-56.	11, 13
Y	US 4,999,344 A (JETT-TILTON et al) 12 March 1991, col. 8, lines 50-68.	11

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	T	later documents published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance	X	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
B earlier document published on or after the international filing date	Y	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combinations being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	A*	document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means		
P documents published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

07 AUGUST 1997

Date of mailing of the international search report

24 SEP 1997

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

SHARIDAN CARRILLO

Telephone No. (703) 306-1876

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/09604

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐

The additional search fees were accompanied by the applicant's protest.

☒

No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/09604

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claim(s) 1-10 and 14-15, drawn to a method of detecting gynecological carcinomas.

Group II, claim(s) 11-13, drawn to a method of extracting lysophosphatidic acid.

The inventions listed as Groups I and II do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

The claims of these two groups are directed to different inventions which are not linked to form a single general concept. The claims in the different groups do not have in common the same or corresponding "special technical feature". Specifically, Group I recites a method of detecting carcinomas by preparing a lipid extract, separating the phospholipid fractions from the extract, and detecting the presence of lysophosphatidic acid. Group I does not include the special technical feature of Group II, namely the step of extracting lysophosphatidic acid using an acid/organic solvent composition.